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An immunohistochemical study of enteric nervous system in the chick model

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Abstract

The chick model is a useful research tool to investigate the development of the enteric nervous system (ENS). Recognition of appropriate markers for detection of chick enteric ganglia will allow better utilization of this model to study abnormalities of the ENS. This study aimed to validate a set of antibodies for avian ENS studies on wax sections. The specimens were taken from jejunum and colorectum of early post-hatching chicks, fixed in 4% buffered formaldehyde and stained using haematoxylin and eosin (H&E). Glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), synaptophysin and S-100 immunohistochemical biomarkers were employed on paraffin-embedded blocks to identify enteric ganglia. The immuno-reactivity scoring was recorded using a semi-quantitative four-tiered system (0, 1+, 2+, and 3+).

In jejunum specimens, the immune-reactivity of GFAP was significantly higher than both synaptophysin (p=0.001) and S-100 (p=0.001). There was also a significant difference (p=0.03) between the immune-reactivity induced by NSE and S-100 in the jejunum samples. Significant differences were observed between GFAP immuno-reactivity and both synaptophysin and S-100 (p=0.013; and p =0.005, respectively) in the samples collected from colorectum. The level of immuno-reactivity between NSE and both synaptophysin and S-100 biomarkers in the colorectal specimens were also different significantly (p=0.02 and 0.007, respectively). The results of the present work showed that GFAP and NSE biomarkers can be used with high immuno-reactivities to examine the chick enteric ganglia as an appropriate animal model in ENS developmental disorders.

Keywords: Enteric Nervous System, Immunohistochemistry, Chick

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Introduction

The enteric nervous system (ENS) cells (neurons and glia) arise from neural crest and migrate along the gastrointestinal (GI) tract in precise pathways to form submucosal and myenteric plexuses (Metzger, 2010 and Burns, 2005). Any disturbances in these pathways during embryonic development, can lead to congenital GI tract disorders, dysmotilities and serious diseases (Gershon and Ratcliffe, 2004). Three types of miss-migrating induced dysmotilities have been recognized in human being characterized by aganglionosis, hypoganglionosis and hyperganglionosis (Kapur, 2000 andFenoglio-Preiser *et al*., 2008).

Hirschsprung's disease or aganglionic megacolon is the most important dysmotility of colon that is characterized by abdominal distention, anorexia, and lethargy. This abnormality is typically diagnosed in infancy with a morbidity of 1: 5000 live births. The disease is caused by the failure in normal cranial-to-caudal migration of neural crest cells during development resulting in the congenital absence of the ganglion cells in both the myenteric and submucosal plexuses of distal colon (Fenoglio-Preiser *et al*., 2008). Molecular and biochemical mechanisms underlying pathogenesis of Hirschsprung's disease are complex and have not been fully understood yet (Amiel *et al*., 2007). So, the development and introduction of an animal model that can reflect the human GI dysmotilities is essential for diagnosis, prevention and treatment of the diseases. There is evidence that the chick model can be a good research tool for studying development and abnormalities of the intestinal nervous system (Parisi Salvi*et al*., 2004; Goldstein and Nagy, 2008 and Doyle *et al*., 2004). Many investigators have used this model for study of the cellular origins and migratory pathways of enteric neural crest-derived cells to disclose the molecular mechanisms regulating ENS development (Goldstein and Nagy, 2008).

Haematoxylin and eosin (H&E) staining technique is not considered as a reliable tool for evaluation of ENS ganglia if it is used alone because this technique is not able to discriminate neural cells from endothelial cells, fibroblasts, histocytesor lymphoblasts (Kapur, 2006 andKarim*et al*., 2006). This limitation has fueled attempts to develop the new immunohistochemical markers that can simplify the identification of enteric ganglia. However, despite many studies on immunostaining patterns of various neural and non-neural antigens in Hirschsprung's disease, no single antibody or panel of antibodies have received general agreement in the current method of disease diagnosis (Fenoglio-Preiser *et al*., 2008).

Different antibodies used for diagnosis of Hirschsprung's disease in human include neuron specific enolase (NSE), S-100, glial fibrillary acidic protein (GFAP) and synaptophysin. NSE immunoreacts with nerves and glial cells, but S-100 can identify Schwann cells and glia, and GFAP is able to stain glia. Among these, it seems that NSE is the best, because of a higher immuno-reactivity (Fenoglio-Preiser *et al*., 2008 and Yanlei Huang *et al*., 2011). GFAP and S-100 have been used to investigate the patterning of the ENS in chick gut (Balaskas and Gabella, 1997). However, it is known that the sensitivity and specificity of ENS detection by these biomarkers are variable because of the probable differences between human and avian antigenic epitopes (Balaskas and Gabella, 1997; Maruccio*et al*., 2008 and Ruiz *et al*., 2005). Therefore, recognition of appropriate markers for detection of chick enteric ganglia will allow better utilization of this model to study abnormalities of the ENS (Asia *et al*., 1997). This study sought to examine the immuno-reactivity of current available immunohistochemical biomarkers in early post-hatching chick gut in order to facilitate the identification of enteric ganglia in this experimental model.

Materials and Methods

Animals

Ten 3 day-old male newborn chicks (*Gallus domesticus,* White Leghorn) that hatched in our own laboratory were used in the study. The eggs were provided from a merchandized center and then incubated at temperatures between 37.5 to 37.6$^{\circ C}$ and 70% humidity in a forced air incubator with automatically control of temperature and humidity. The reason for using 3-day-old chicks was to allow the total expression of biomarker targets in the ENS (Li et al., 2001 and Rouleau *et al*., 2009). All animals were treated according to the standard procedures outlined by Institutional Ethical Committee.

Tissue preparation

Chicks were euthanized by CO_2 gas and then the abdomen was immediately opened by an incision made on ventral midline. After gross examination of GI tract, the segments were collected from both jejunum (as representative of foregut) and colorectum (as representative of hindgut). The gut specimens were cut into 10 mm isolated rings and then each cut was divided into two different sections, one longitudinal and one transverse section. The tissue sections were fixed by 4% formal dehydein 0.1 M phosphate-buffered saline (PBS) solution. The fixed tissues were dehydrated by graded concentrations of ethanol and embedded in paraffin wax, and then they were stained with H&E as described previously (Aitken, 1958). A pathologist reviewed each slide twice, and the likely existence areas of myenteric or submucosal plexuses were marked on the back of the slides.

Immunohistochemical study

Paraffin-embedded blocks were cooled in an ice-water mixture for 30 min before sectioning. 4 µm sections were cut and placed on slides. After a brief drying period of approximately 15 min, the sections were heat-fixed to the slide at 37$^{\circ C}$.The sections were deparaffinized and rehydrated in graded ethanol concentrations. The prepared slides were stained immunohistochemically according to instructions of kits manufacturer (DAKO, Glostrup, Denmark) by S-100 (polyclonal rabbits anti-human antibody; Code No: Z0311), NSE (monoclonal mouse anti-human antibody; Code No: IR612), GFAP (polyclonal rabbits anti-human antibody; Code No.: Z0334) and synaptophysin (monoclonal mouse anti-human antibody; Code No.: IS776). Briefly, antigen retrieval was accomplished by using heat and citrate buffer for 10 min. Then 3% H_2O_2 and pure methanol were added for 5 min and sections were washed with distilled water. The primary antibody (with negative control) was added for 10 min and washing was accomplished. The secondary antibody (biotinylated link) was added to the sections for 10 min and then PBS washed them. The slides were stained by addition of diaminobenzidine as chromogensubstrate for 10 min. Totally 80 immunohistochemically stained slides were prepared in this study; 40 slides for jejunum and 40 slides for colorectum. 10 samples were analyzed per gut region and per marker. In addition, two paraffin blocks of normoganglionic human jejunum and colon prepared during 6 last months were taken from a medical center and stained with NSE according to DAKO's instruction. The myenteric and submucosal plexuses of these slides were used as positive controls.

In double-blinded microscopic observation on H&E slides, the points that had been suspected as ENS regions were matched with immunohistochemical slides and then assessed microscopically with magnifications of 100× and 400×. With magnification of 400× (40× objective in conjunction with a 10× ocular), the slides were scored as 0 for negative immuno-reactivity, + for weak immunereactivity, ++ for intermediate immuno-reactivity and +++ for good immuno-reactivity. Owing the red blood cell nucleation in avian, discrimination of nerve plexuses from red blood cells in both H&E and

immunohistochemical staining is usually complicated, but changing the magnification and using of microscopy micro screw solved the problem.

Statistics

The raw data were represented as mean ± SEM. The statistical significance of differences was calculated with one way analysis of variance (ANOVA) followed by Bonferroni t-testusing BioStatprogram, version 2008. A *p* values less than 0.05 were considered significant.

Results

The photomicrographs taken from different parts of 3- day chicken gut are shown in Figure 1. This picture illustrates the microscopic view of enteric nervous ganglia in newborn chick stained with H&E as well as immune-histochemical techniques using different biomarkers. The statistical analyses of the immuno-reactivity of enteric nervous ganglia to different biomarkers are summarized in Figure 2. There was a significant difference in immuno-reactivity between slides provided from jejunum by different biomarkers as determined by one-way ANOVA (p=0.002). GFAP and NSE showed the highest immuno-reactivity in the jejunum pieces compared to synaptophysin and S-100 (Figure2). Although the immuno-reactivity of GFAP was higher than NSE in the jejunum samples (2.10±0.18 and 1.70±0.26, respectively), the difference was not statistically significant. The immuno-reactivity values obtained from GFAP staining in jejunum slices were significantly higher than those shown by synaptophysin and S-100 (2.10±0.18 vs. 1.1±0.18, p=0.001; 2.10±0.18 vs. 0.80±0.29, p =0.001, respectively). A significant difference was also illustrated between the immuno-reactivity of NSE and S-100 in the jejunum cut (1.70±0.26 vs. 0.80±0.29, p =0.033).

The highest immuno-reactivity was found for GFAP and NSE in slides provided from colorectal specimens (2.00±0.21 and 1.90±0.18, respectively), but the difference between GFAP and NSE was not statistically significant. There were significant differences between GFAP immuno-reactivity with both synaptophysin (2.00±0.21 vs. 1.20±0.20 with p =0.013) and S-100 biomarker (2.00±0.21 vs. 0.90±0.28 with p =0.005). The immuno-reactivity induced by NSE in colon cut was significantly bigger than those obtained from synaptophysin (1.90±0.18vs. 1.20±0.20, p =0.018) and S-100 (1.90±0.18 vs. 0.90±0.28, p =0.007). However, there was not statistically significant difference between synaptophysin and S-100 immunoreactivities in the cuts taken from colon (Figure 2). The immuno-reactivity induced by different biomarkers in colorectal samples was not significantly different with those obtained from jejunum slides.

Discussion

Developmental disorders of the ENS can result in different abnormalities including Hirschsprung's disease in humans and congenital colonic aganglionosis in foals (Gershon and Ratcliffe, 2004 and Porter *et al*., 2007). The intestinal neuronal dysplasia manifested by hyperganglionosis and the presence of giant and ectopic ganglia is another congenital disorder of gastrointestinal motility in humans (Ure *et al*., 1994). Many studies are conducted to show the molecular and biochemical mechanisms underlying these abnormalities, but the pathophysiology of these disorders have not been fully understood. It seems that any advances in characterization and introduction of a valid animal model may hold the key to future insights. Several investigators have focused on avian gastrointestinal tract as a suitable animal model to examine such developmental disorders because of well characterization of the migration and patterning of ENS (O'Donnell and Puri, 2009). Immunohistochemical staining is known as the key diagnostic method to identify enteric ganglionic cells. Several immunohistochemically detectable antigens have been introduced to identify ENS, however, none of the current available markers

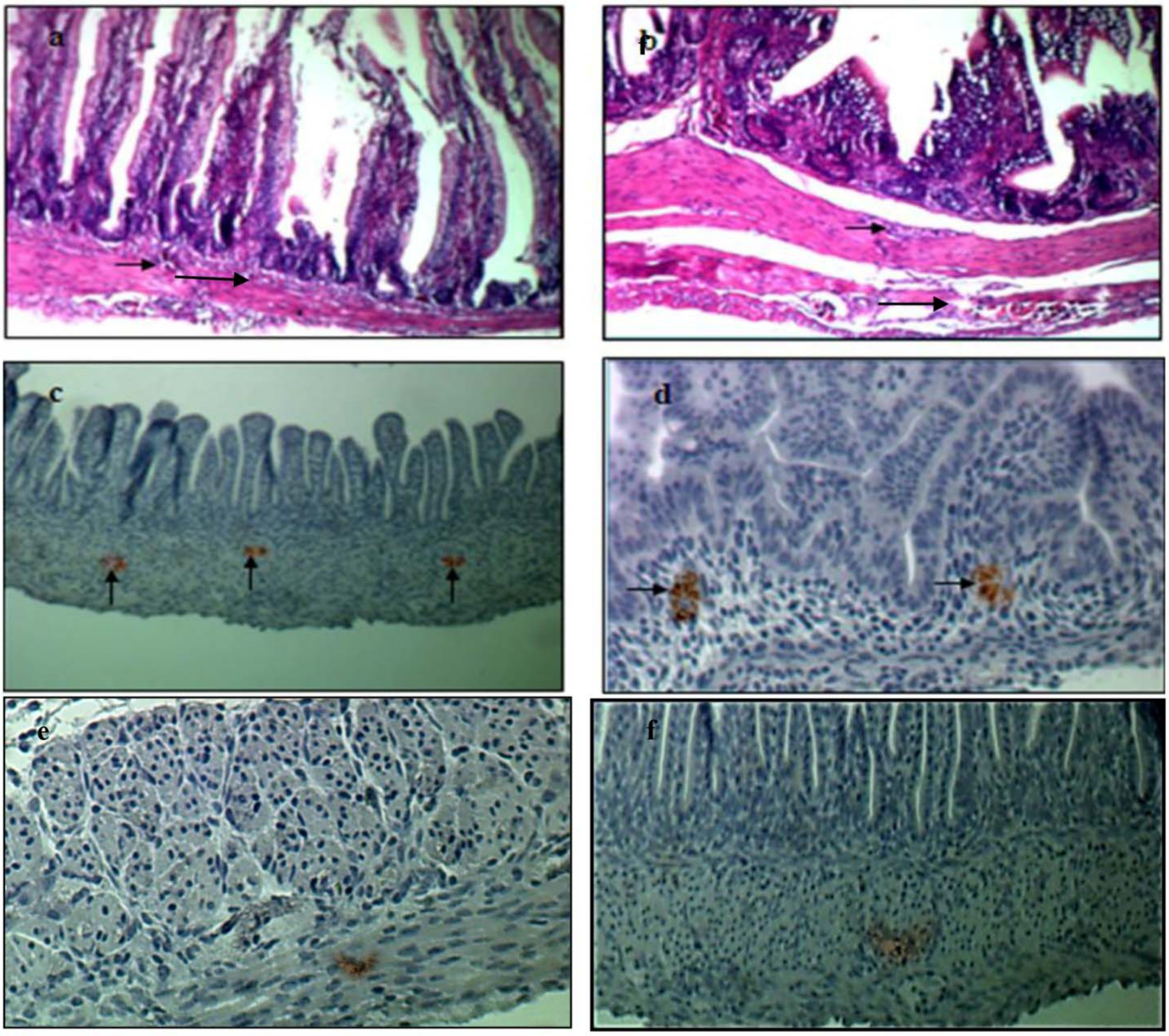


Figure 1. Microscopic view of enteric nervous ganglia in newborn chick.(a) H&E staining of jejunum (original magnification, ×100) (b) H&E staining of colon (original magnification, ×100) (c) Immunohistochemical staining of jejunum by GFAP marker that was able to identify enteric ganglia with a high intensity (original magnification, ×100) (d) Immunohistochemical staining of colon by NSE that is shown the ganglia with a good intensity (original magnification, ×400) (e) Immunohistochemical staining of jejunum by synaptophysin that its immune-reactivity is weak (original magnification, ×100) (f) Immunohistochemical staining of colon by S-100 with a low intensity in colon (original magnification, ×400). Both H&E and immunohistochemistry slides were prepared from the same samples. The arrows indicate the enteric nervous ganglia.

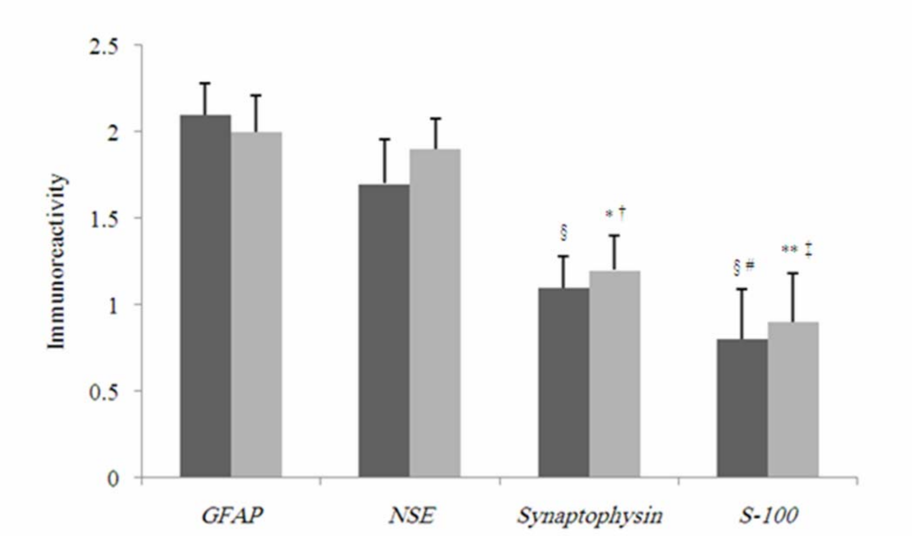


Figure 2.Immunoreactivity of enteric nervous ganglia obtained from the application of GFAP, NSE, synaptophysin and S-100 biomarkers in the jejunum and colon specimens of 3-day chicks. Immunoreactivity was scored by a semi-quantitative four-tiered system (0, 1+, 2+, and 3+). Black bar shows the immunoreactivity in jejunum slides and the grey bar indicate the immunoreactivity of colon cuts. The values are expressed as mean plus Error bar standard error of the mean. § $p < 0.01$ vs. GFAP in jejunum. #$p< 0.05$ vs. NSE in jejunum.* $p < 0.05$ vs. GFAP in colon. $p < 0.01$ vs. GFAP in colon. † $p < 0.05$ vs. NSE in colon. ‡ $p <0.01$ vs. NSE in colon.**

has been considered as a gold standard test for investigation or diagnosis of ENS disorders (Fenoglio-Preiser *et al*., 2008). In response to the need for introducing a suitable animal model for detection of enteric ganglion cells, the present study sought to compare the immuno-reactivity of four routine biomarkers in avian gut. Our work demonstrates that ganglion cell detection and its staining with GFAP and NSE were better than synaptophysin and S-100 in the chick gut. The results of this study indicated that GFAP and NSE markers could show high amount of immune reactivities for detection of avian enteric ganglia as an animal model for ENS developmental disorders. It is also found that unlike human enteric ganglia, S-100 has a poor immuno-reactivity in avian ENS suggesting that S-100 biomarker should not be considered as a suitable marker for experimental studies of avian ENS.

GFAP is an intermediate filament protein expressed by different cells of the nervous system including astrocytes. This proteinis thought to be involved in the maintenance of astrocyte mechanical strength, as well as the shape of cells. However, its exact function has not been fully understood (Dabbs, 2006). NSE is found in a variety of normal and neoplastic neuroendocrine cells with the largest amount in the brain tissue (Dabbs, 2006). Synaptophysin as a glycoprotein is an integral part of the neuroendocrine secretory granule membrane, which its presence in a variety of neuroendocrine tumors has been recognized by monoclonal antibody (SY38) (Dabbs, 2006). S-100 is a low molecular weight protein that is widely distributed in human tissues. This marker is generally used for diagnosis of different tumors including malignant peripheral nerve sheath tumors, schwannomas, paraganglioma stromal cells, histiocytoma, clear cell sarcomas and melanoma. It is known as an appropriate marker for staining of Schwann and glial cells (Dabbs, 2006).

Recent works conducted by different research centers have given a body of information about development and structure of ENS in normal or disturbed circumstances. Developing immunohistochemical methods have provided many opportunities for histopathological studies to diagnose the ENS defects with high sensitivity and specificity. However, despite many studies on immunostaining patterns of various neural and non-neural antigens, there is controversy in

the use of any biomarker to analyze the malformations or various defects in ENS. It seems that the staining of immunoreactions depends on tissue species and antiserum quality. Memarzadeh *et al* in a comparative study between IHC and H&E staining methods found that both Cathepsin D and CD56 markers can be the best diagnostic panel for detection of ganglion cells if they are used together (Memarzadeh *et al*., 2009). However, other study showed that immunohistochemical method using bcl-2 could provide a good marker for identification of ganglion cells in the ENS (Wester *et al*., 1999).

Park *et al* reported that the interpretation of the enteric nervous plexuses in the transitional zone and the detection of immature neuronsin distinguishable from glial cells, are the main complexity in diagnostic methods. So, they found S-100 protein, synaptophysin and CD56 as helpful diagnostic adjunct to describe the size of the enteric ganglia. They also introduced bcl-2 as a suitable biomarker for detection of immature enteric ganglion cells (Park et al., 2005). Other study showed that synaptophysin immuno-reactivity can be a suitable tool for detection of enteric ganglia (Dzienis-Koronkiewicz *et al*., 2005). However, Karim *et al* demonstrated that ret oncoprotein in immuno-reactivity has a high sensitivity in identification of enteric ganglion cells (Karim*et al*., 2006). While, Kawana *et al* reported that immunohistochemistry for GFAP can be an excellent diagnostic tool in ENS plexuses (Kawana*et al*., 1989) and Robey *et al* suggested that NSE and S-100 markers are of value in demonstrating enteric ganglion cells (Robey*et al*, 1988). To optimize the method of diagnosis in ENS, Cecilia *et al* have introduced an IHC panel of antigens for intestinal dysmotility including PGD 9.5, NSE, MAP-2, NCAM, NGFR, neuropeptide Y, neurofilament protein, synaptophysin, RET, acetyl cholinesterase, c-kit, VIP, substance P, S-100, GFAP and actin (Fenoglio-Preiser *et al*., 2008). Additionally, Doyle*et al* in an experimental model of Hirschsprung's disease examined the molecular mechanisms governing migration and patterning in the avian hindgut ENS, and they found that HNK-1 antigen can be a promising immunohistochemistry target to study avian ENS (Doyle *et al*., 2004).

Owing to introducing a wide diversity of diagnostic biomarkers by many scientific centers, it is difficult to find the best immunohistochemical panel for detection of enteric ganglion cells. Most of the above mentioned markers are generally used in research studies and few of them are applied generally in histopathology tests fori dentification of enteric ganglia. Because of species variation, it is obvious that some antibodies used in medicine may not be able to detect the avian tissue antigens (Balaskas and Gabella, 1997). Furthermore, there are some limitations in the use of antibodies specific for avian tissues because of economical reasons and many limitations to access. However, GFAP, NSE, synaptophysin and S-100 are the most and well-known markers that are widely applied for identification of enteric ganglia (Fenoglio-Preiser*et al*., 2008). It is known that these biomarkers are able to induce good reactivity with the avian ENS in some way because of the similarity of epitopes between human and avian intestinal tissue (Balaskas and Gabella, 1997).

In conclusion, the results of the present study showed that GFAPand NSE could be reliable immunohistochemistry markersto detect avian enteric ganglia as an animal model for ENS developmental disorders. We also found that unlike human enteric ganglia, S-100 has a poor immuno-reactivity in avian ENS suggesting that this biomarker should not be considered as a suitable marker in the studies of avian ENS. So, this work strongly suggests that the immuno-reactivity of GFAP in avian ENS is higher than those shown in human.

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مطالعه ایمونوهیستوشیمیایی سیستم عصبی روده ای در مدل مرغ

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چکیده

مرغ به عنوان یک مدل حیوانی مناسب در مطالعه تکامل سیستم عصبی روده‌ای مطرح می‌باشد. شناسایی مارکرهای مناسب جهت تشخیص گانگلیون‌های روده‌ای مرغ زمینه را برای استفاده بهتر از این مدل حیوانی در مطالعات ناهنجاری‌های سیستم عصبی روده‌ای فراهم خواهد نمود. هدف از پژوهش حاضر، ارزیابی میزان واکنش‌پذیری ایمنی چند آنتی‌بادی با سیستم عصبی روده‌ای مرغ در بلوک‌های پارافینی می‌باشد. بدین منظور، قطعات ژژونوم و کولورکتوم از جوجه‌های ۳ روزه اخذ و در فرمالین بافر ۴٪ تثبیت شد و با هماتوکسیلین و ائوزین رنگ‌آمیزی گردید. به منظور تشخیص سیستم عصبی روده‌ای در بلوک‌های پارافینی با روش ایمونوهیستوشیمی از آنتی‌بادی‌های ضد Glial Fibrillary Acidic Protein(GFAP)، Neuron Specific Enolase(NSE)، Synaptophysinو S-100 استفاده شد. برای ارزیابی میزان واکنش‌پذیری ایمنی از سیستم امتیازدهی (۰، ۱+، ۲+ و ۳+) بهره گرفته شد.

در نمونه‌های ژژونوم، میزان واکنش‌پذیری GFAP به شکل معنی‌داری ($p = ۰/۰۰۱$) بیشتر از Synaptophysin و S-100 بود. در بافت ژژونوم واکنش‌پذیری ایمنی NSE نیز به شکل معنی‌داری بیشتر از S-100 بود ($p = ۰/۰۳$). در بافت کولورکتوم، واکنش‌پذیری ایمنی GFAP به شکل معنی‌داری بیشتر از Synaptophysin ($p = ۰/۰۱۳$) و S-100 ($p = ۰/۰۰۵$) بود. واکنش‌پذیری ایمنی NSE نیز به صورت معنی‌داری بیشتر از Synaptophysin ($p = ۰/۰۲$) و S-100 ($p = ۰/۰۰۷$) بود.

نتایج تحقیق حاضر گویای آن بود که آنتی‌بادی‌های ضد GFAP و NSE واکنش‌پذیری بالایی جهت تشخیص گانگلیون‌های سیستم عصبی روده‌ای مرغ که به عنوان مدل حیوانی مناسب جهت مطالعه ناهنجاری‌های تکاملی سیستم عصبی روده‌ای مطرح می‌باشد،برخوردار هستند.

واژگان کلیدی: سیستم عصبی روده‌ای، ایمونوهیستوشیمیایی، مرغ